

Nucleotides and Nucleotide Sugars in Developing Maize Endosperms¹

Synthesis of ADP-Glucose in *brittle-1*

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As part of an in vivo study of carbohydrate metabolism during development of *Zea mays* L. kernels, quantities of nucleotides and nucleotide sugars were measured in endosperm extracts from normal, the single-mutant genotypes *shrunk-1* (*sh1*), *shrunk-2* (*sh2*), and *brittle-1* (*bt1*), and the multiple-mutant genotypes *sh1bt1*, *sh2bt1*, and *sh1sh2bt1*. Results showed that *bt1* kernels accumulated more than 13 times as much adenosine 5' diphosphoglucose (ADP-Glc) as normal kernels. Activity of starch synthase in *bt1* endosperm was equal to that in endosperm extracts from normal kernels. Thus the ADP-Glc accumulation in *bt1* endosperm cells was not due to a deficiency in starch synthase. ADP-Glc content in extracts of *sh1bt1* endosperms was similar to that in *bt1*, but in extracts of the *sh2bt1* mutant kernels ADP-Glc content was much reduced compared to *bt1* (about 3 times higher than that in normal). Endosperm extracts from *sh1sh2bt1*, kernels that are deficient in both ADP-Glc pyrophosphorylase (AGPase) and sucrose synthase, had quantities of ADP-Glc much lower than in normal kernels. These results clearly indicate that AGPase is the predominant enzyme responsible for the in vivo synthesis of ADP-Glc in *bt1* mutant kernels, but Suc synthase may also contribute to the synthesis of ADP-Glc in kernels deficient in AGPase.

A number of maize (*Zea mays* L.) endosperm mutants that affect the quantity and quality of carbohydrates in the endosperm, as well as kernel development and morphology, have been identified and extensively studied (Shannon and Garwood, 1984). Mutants such as *waxy* (*wx*), *amylase extender* (*ae*), and *sugary-1* affect the quantity and branching characteristic of kernel polysaccharides (Shannon and Garwood, 1984). Several other mutants involve a defect in the metabolism of sugars, resulting in the accumulation of Suc and the reduction in starch. For example, *sh1* kernels are deficient in the major Suc synthase enzyme (Chourey and Nelson, 1976), and *sh2* and *brittle-2* mutant kernels are both deficient in AGPase (Preiss, 1991). The genetic lesion of *bt1*, another high-sugar/low-starch mutant, is not as well defined. Early screening of developing *bt1* kernels for the activity of various enzymes of carbohydrate metabolism and starch biosynthesis failed to identify

a specific enzyme lesion. Developing *bt1* kernels, compared to normal, have been reported to be low in a starch granule-bound phospho-oligosaccharide synthase (Pan and Nelson, 1985) and starch debranching enzyme but twice as high in AGPase (Doehlert and Kuo, 1990).

Sullivan et al. (1991) reported the isolation and analysis of the *Bt1* gene from maize and showed that the protein with greatest similarity to the *Bt1*-encoded protein is a yeast adenylate translocator. Li et al. (1992) showed that the in vitro translated *Bt1* gene product could be imported into chloroplasts, where it was processed and localized to the inner envelope membrane. We have isolated amyloplast membranes from normal and *bt1* kernels and showed that the four most abundant amyloplast membrane polypeptides (38–44 kD) from normal kernels were specifically recognized by antibodies raised against BT1 (Cao et al., 1995). In contrast, the four abundant BT1 immunoreactive polypeptides were missing from amyloplast membranes isolated from *bt1* mutant kernels. Liu et al. (1992) reported that amyloplasts isolated from young maize kernels effectively take up ADP-Glc for starch synthesis, but amyloplasts from *bt1* are only 25% as active in ADP-Glc uptake and incorporation into starch as amyloplasts from normal maize endosperm. The precise function of the *Bt1* gene product is unknown, but it must be important to starch accumulation, since starch content of *bt1* kernels is only about 20% of that in normal maize endosperm (Tobias et al., 1992).

As part of a continuing in vivo/genetic approach to the study of carbohydrate metabolism in normal and starch-deficient maize endosperm mutant genotypes, we discovered that *bt1* kernels accumulate very high levels of ADP-Glc. In this paper we report the nucleotide composition of endosperm extracts from normal and mutant genotypes. Results of a study designed to determine the enzyme(s) responsible for the in vivo synthesis of ADP-Glc in *bt1* kernels are also reported.

Abbreviations: ADP-Glc, adenosine 5' diphosphoglucose; AGPase, ADP-Glc pyrophosphorylase; *bt1*, *brittle-1*; DPP, days postpollination; SBE, starch branching enzyme; *sh1* and *sh2*, *shrunk-1* and *shrunk-2*, respectively; UDP-Glc, uridine 5' diphosphoglucose; UGPase, UDP-Glc pyrophosphorylase; UPTG, UDP-Glc:protein transglucosylase.

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MATERIALS AND METHODS

Plant Material

The normal maize (*Zea mays* L.) inbred W64A and the endosperm mutant genotypes *sh1* and *bt1* in a near isogenic W64A background were grown in the field at the Russell E. Larson Agricultural Research Farm (Center County, PA) during the summer of 1994. Additional plants for the single mutants *bt1*, *sh1*, and *sh2* and the multiple mutants *sh1bt1* and *sh2bt1*, also in a near-isogenic W64A background, were grown in 20-L plastic pots containing a loose growing medium consisting of two parts peat, two parts perlite, and one part soil. The *sh2* plants were grown in the spring of 1993, and the other single-, double-, and triple- (*sh1sh2bt1*) mutant genotypes were started in the greenhouse in late spring of 1994 and then transferred outside the greenhouse for continued growth. High-intensity sodium lamps were used in the greenhouse to extend the daylength to 16 h. For the experiments comparing nucleotide levels in endosperm tissue containing different dosages of the *Bt1* gene and different developmental ages (10 and 20 DPP), the plants were grown in the greenhouse during the spring of 1993. For the dosage experiment, cross-pollinations and reciprocal cross-pollinations were made between W64A and *bt1*. All ears were hand-pollinated and harvested 20 DPP (except for the developmental age experiment). For plants grown in 1993, kernels were cut from the cobs leaving approximately the lower one-fifth on the cob. The pericarp and embryo were removed from each kernel, and the endosperm was quickly frozen in liquid nitrogen and freeze dried. To reduce the time between ear removal from the plant and freezing, in 1994, the kernels were cut from the ear, quickly frozen in liquid nitrogen, and freeze dried prior to removing the embryos. The dried endosperms and pericarps were pulverized in a mortar and pestle, taking care not to finely pulverize the pericarp. Most of the pericarp was removed by sifting the powdered endosperm through a 40-mesh stainless steel sieve (Collector Tissue Sieve, E-C Apparatus Corp., Thomas Scientific, Swedesboro, NJ). Although the "endosperm" samples from the kernels harvested in 1994 may have contained some pericarp tissue, in our opinion the advantage of the increased speed of freezing the samples outweighed the disadvantage of the slight pericarp contamination.

Identification of Double and Triple Mutants by Dot Blot and Embryo Culture

To isolate the double-mutant genotypes, homozygous *sh1* and *sh2* plants were pollinated with pollen from homozygous *bt1* plants. The *sh1*, *sh2*, and *bt1* plants were all in a near-isogenic W64A inbred background. To produce the triple-mutant genotype *sh1sh2bt1*, a *sh1sh2* double-mutant plant (the *sh1sh2* kernels, a gift from William Tracy, University of Wisconsin, Madison, were not isogenic with W64A) was pollinated with pollen from a homozygous *bt1* plant. F_1 seeds resulting from these crosses were planted in 20-L containers as described above, and the plants were grown in the greenhouse in the spring of 1994. The F_1 plants were self-pollinated and the F_2 ears were harvested

20 DPP. Intact kernels were removed from the ears and surface sterilized for 15 min in a 20% chlorine bleach solution containing two drops of Tween 20 per 100 mL of solution. The kernels were rinsed in sterile water, and the upper one-third of at least 100 kernels from each ear was excised for immunoassay by tissue printing. For the immunoassay, the cut surface of each endosperm was blotted onto two (for the double-mutant selections) or three (for the triple-mutant selections) nitrocellulose membranes (see below). The rest of each kernel, including the embryo, was placed in individual wells of a sterile ELISA plate. Each well contained one to two drops of sterile water. The ELISA plates containing the kernel pieces were closed and stored in the cold for about 24 h until the results of the dot blot immunoassay were complete. Care was taken to maintain the identity of the residual kernel pieces with the dot blots. Putative *sh1bt1* kernels were identified by the absence of BT1 antibody-reacting proteins (the BT1 antibody was a gift from Thomas Sullivan, University of Wisconsin, Madison) on one membrane and the absence of Suc synthase (SH1) antibody-reacting proteins (the SH1 antibody was a gift from Prem Chourey, U.S. Department of Agriculture/University of Florida, Gainesville) on a second membrane. Likewise, the *sh2bt1* and *sh1sh2bt1* kernels were identified as those missing the SH2 (antibodies to the SH2 polypeptide were a gift from L. Curtis Hannah, University of Florida, Gainesville) and BT1 polypeptides and those missing the SH1, SH2, and BT1 polypeptides, respectively. Theoretically, one-sixteenth of the F_2 kernels on F_1 plants from the *sh1* \times *bt1* or *sh2* \times *bt1* crosses should be double mutants and one-sixty-fourth of the F_2 kernels on the *sh1sh2* \times *bt1* F_1 plants should be the triple-mutant genotype. The embryos from 22 putative *sh1bt1*, 18 *sh2bt1*, and 3 *sh1sh2bt1* kernels were excised and cultured in vitro according to the procedure of Smith (1992). The cultured embryos were allowed to germinate in the light at 22°C for 14 d. The established seedlings were then removed from the tubes and transplanted to pots containing Fafard Canadian Growing Mix No. 2 (Conrad Fafard Inc., Agawam, MA). After 14 d of growth in the culture room, the seedlings were transplanted to 20-L plastic pots and growth continued in the greenhouse and then outdoors. Each plant was self-pollinated, and two-thirds of the ear were harvested 20 DPP for analysis. The genotype of each ear was confirmed by dot blot immunoassay and by enzymatic analyses, and the remaining one-third of each ear was allowed to develop to maturity. Of all of the plants tentatively identified as multiple mutants, 19 of 22 were *sh1bt1*, 9 of 18 were *sh2bt1*, and 1 of 3 was *sh1sh2bt1*.

The method for tissue printing was adapted from the procedure described by Campillo (1992). Briefly, the nitrocellulose membrane was soaked in 0.2 M CaCl_2 for 20 min with shaking and dried on 3-MM Whatman chromatographic paper. Then the membrane was printed with endosperm tissue and blocked in a tray for 1 to 2 h with the blocking buffer (5% [w/v] nonfat dry milk, 1% [w/v] BSA, 1 M Gly, and 0.02% sodium azide in TTBS buffer [20 mM Tris-base, pH 7.5, 150 mM NaCl, and 0.05% Tween 20]) by gentle shaking. The blocking buffer was replaced with

TTBS buffer containing 1% nonfat dry milk, 0.02% sodium azide, and the primary antibody and gently shaken for 1 to 2 h. After the printed membrane was washed twice with TTBS buffer for 5 min each time, it was incubated in the blocking buffer containing the secondary antibody for 1 to 2 h with gentle shaking. The secondary antibody for SH1 consisted of 10 μ L of goat anti-mouse IgG-alkaline phosphatase conjugate (Sigma catalog No. A5153) in 10 mL of blocking buffer. For SH2 and BT1, 10 μ L of goat anti-rabbit IgG-alkaline phosphatase conjugate (Sigma catalog No. A9919) was added to 10 mL of blocking buffer. The membranes were then washed twice with TTBS buffer for 10 min each time and once with TBS (TTBS buffer without Tween 20) for 10 min. The reaction was then detected with 0.015% (w/v) 5-bromo-4-chloro-3-indoyl phosphate and 0.03% (w/v) nitroblue tetrazolium in AP buffer (0.1 M Tris base, 0.1 M NaCl, and 5 mM MgCl₂, pH 9.5). The reaction was stopped by quickly washing the membrane with distilled water. Finally, the membrane was sealed in a plastic bag and stored in a cool place for future reference.

Enzyme Extraction and Assay

Fifty milligrams of freeze-dried endosperm tissue were homogenized in 2 mL of extraction buffer (10 mM Tricine, pH 7.2, 1 mM DTT, 1 mM EDTA, 0.5 mM PMSF) and then centrifuged at 20,000g for 20 min in the cold (4°C). The crude supernatant was used for the assay of AGPase, UGPase, soluble starch synthase, and SBE. The pellet was washed once with extraction buffer and then suspended in extraction buffer, and an aliquot was removed for assay of starch granule-bound starch synthase. Prior to the assay of Suc synthase, an aliquot of the supernatant was desalted by centrifugal filtration on Sephadex G-25 columns equilibrated with extraction buffer. The procedures described by Echeverria et al. (1988) were followed for assay of AGPase, UGPase, starch synthase (including soluble and granular bound), and SBE, except that the activity of SBE was measured using nonradioactive Glc-1-P as a substrate and the released Pi was determined as described by Lanzetta et al. (1979). The Pi released in reactions without added phosphorylase was subtracted from that released in the complete reaction mixture. Suc synthase activity was measured in the synthetic direction according to the procedure of Guy et al. (1992). Soluble protein in the enzyme extracts was determined using the Bio-Rad protein assay based on the Bradford method. BSA was used as a standard.

Metabolite Extraction

Equal weights of pulverized, sifted endosperm from three *sh1bt1* ears were combined to produce composite sample 1 and sifted endosperm from another three ears combined for composite sample 2. Likewise, for the two *sh2bt1* composite samples, equal weights of pulverized, sifted endosperm from four and three ears were combined. Pulverized, sifted endosperm from a single ear was used for the *sh1sh2bt1* samples. Pulverized endosperm from several ears were combined for the composite samples of normal (W64A), *bt1*, *sh1*, and *sh2*.

Duplicate 0.5-g samples of freeze-dried endosperm tissues from the single-mutant and triple-mutant genotypes and from the two composite samples of the double-mutant genotypes were homogenized in a 10-mL Potter-Elvehjem-type homogenizer with a Teflon pestle (Thomas Scientific) with 3 mL of 0.8 M HClO₄ in a frozen slurry state. The ice slurry was obtained by mixing dry ice powder with the tissue and HClO₄ prior to homogenization. Then, the homogenates were immediately centrifuged at 2,500g in the cold (4°C) for 10 min, and the pellets were washed twice with cold water (1 mL) each. The combined supernatants (5–6 mL) were collected in 40-mL conical centrifuge tubes and neutralized by addition of an equal volume of cold 0.5 M tri-*n*-octylamine in 1,1,2-trichlorotrifluoroethane. The extract was mixed vigorously for 30 s. Three layers formed during centrifugation at 1,000g (4°C) for 5 min. The intermediate layer solidified during centrifugation, and the upper aqueous layer was withdrawn from the tube. Three to 4 mL of H₂O were added to the residual layers, and after the sample was mixed and centrifuged, the upper aqueous layer was removed and added to the initial aqueous extract. This step effectively removes the perchlorate from the aqueous fraction and adjusts the pH to between 6.0 and 6.5 (Heinrich and Rapoport, 1974). The combined aqueous layers were freeze dried for 24 h. The dried extracts were dissolved in water, and any insoluble residues were removed by centrifugation at 16,000g for 10 min (Eppendorf centrifuge 5415C) at 4°C. The clarified supernatants were retained for metabolite analysis. In a preliminary study we determined the recovery following extraction of added standards, and all metabolite data were corrected based on the percentage recovery of the individual metabolite.

Measurement of Neutral Sugars and Starch

Total reducing sugars and Suc in the extracts were determined by the Nelson test (Hodge and Hoereiter, 1962) and the anthrone test (Ashwell, 1957), respectively. To quantify starch granule number and quantity, the pellet remaining after metabolite extraction was suspended in water and aliquots were removed for counting and quantitative measurement. For quantitative measurement, 10-mL aliquots of the starch suspensions were gelatinized for 30 min in a boiling water bath. After adjustment to a known volume, 1-mL aliquots were added to an equal volume of 1.0 M sodium acetate, pH 4.8, containing 10 units of amyloglucosidase. Following an overnight incubation at 37°C, the digests were heated in a boiling water bath for 1 min to inactivate the enzyme and centrifuged at 20,000g for 10 min. Glc in the supernatants was determined by the Nelson test (Hodge and Hoereiter, 1962). To determine the number of starch granules, aliquots of the starch suspension were appropriately diluted and the number of granules was determined using a Spencer Bright Line Improved Neubauer hemacytometer (0.1 mm deep) (Hausser Scientific, Blue Bell, PA). The numbers of starch granules in random 0.004-mm³ areas of the hemacytometer were counted using a microscope, and the total number of starch granules per sample extract was calculated.

Measurement of Nucleotides and Nucleotide Sugars

The major nucleotides (UMP, UDP, UTP, AMP, ADP, and ATP) and nucleotide sugars (UDP-Glc and ADP-Glc) were separated and quantified by ion-paired HPLC using a reversed-phase Adsorbosphere Nucleotide-Nucleoside 7- μ m column (250 \times 4.6 mm; Alltech Associates, Deerfield, IL). The mobile phases contained: A, 20 mM KH_2PO_4 and 5 mM tetrabutylammonium phosphate, pH 5.0; B, 100% HPLC-grade methanol. The flow rate was 1.5 mL/min. The column was equilibrated with 95% A and 5% B, and then the sample was injected and a linear gradient program from 5% B to 50% B was processed. The 50% B gradient condition was reached in 20 min, and elution continued at the same condition for another 6 min. Each nucleotide was identified by co-migration with commercial standards, and each peak was confirmed by matching the spectrum of each nucleotide peak against that of the standard compounds. The quantity of each nucleotide and nucleotide sugar was determined by comparing the sample peak areas to areas from a standard curve. A Waters Associates HPLC system consisting of dual model 510 pumps, Waters 712 WISP autoinjector, and Waters 996 Photodiode array detector was used for nucleotide analysis. A computer with Waters Millennium 2010 Chromatograph Manager software was connected to the HPLC system to control all of the programs for acquiring and processing the data and calculating the results. The A_{260} was extracted from the photodiode array for quantifying each nucleotide and nucleotide sugar. The relative purity of each peak was determined by comparing the sample nucleotide spectra with standard nucleotide spectra previously stored in the computer. Experiments to determine the effects of *Bt1* gene dosages and developmental ages on nucleotide composition were completed earlier using an older Waters HPLC system, which consisted of Waters dual model 501 pumps, Waters automated gradient controller, Waters model 441 UV absorbance detector (254 nm, 0.2 absorbance units full scale), and the 740 data module.

RESULTS

Recovery Study for Extraction Procedure

In the study reported by Tobias et al. (1992), we encountered difficulties associated with excess salt following neutralization of the HClO_4 extract with K_2CO_3 . The residual KClO_4 salt in the concentrated extracts limited the size of aliquot that could be used for enzymatic analysis of the hexose-*Ps* and triose-*Ps*. Thus, prior to beginning the present study we compared several extraction and neutralization methods. The procedure used in this study was judged to be superior to that used previously (Tobias et al., 1992). The HPLC protocol effectively separated at least 15 compounds that were identified and 5 unidentified peaks (Fig. 1). The identities of the nucleotides were determined by comigration with known standards and confirmed by matching their spectra to those of standards. AMP eluted as the middle peak between two unidentified peaks. The larger unknown peak c has a maximum *A* at 247 nm and its spectrum did not match any of the standard nucleotide

spectra. The UDP, ADP-Glc, and ADP peaks in W64A each had leading shoulders. When these nucleotides were quantified, the integrator was set to exclude the area under the shoulders. The elution time and spectrum of minor peak d matched that of GTP, but since it was very low in all genotypes, the quantities of GTP are not reported. Unknown peak e, a contaminant in the eluant, had a maximum *A* at 280 nm. Recoveries for the uridine and adenosine nucleotides, nucleotide sugars, and NAD ranged between 91 and 112% (Table I). Nucleotide quantities reported were corrected by the respective percentage of recovery. Although data concerning the contents of hexose-*Ps* and triose-*Ps* are not included in this paper, we note that improved recoveries of these metabolites were obtained using this extraction procedure. For example, with the current procedure we obtained 80 and 71% recoveries of added dihydroxyacetone phosphate and glyceraldehyde-3-P, respectively, a significant improvement over the 66 and 28% recoveries of these metabolites reported by Tobias et al. (1992).

Enzyme Activities

Preliminary selections of multiple-mutant genotypes were based on immunoassays of endosperm tissue prints from the segregating F_2 ears harvested 20 DPP (see "Materials and Methods"). Embryos from putative multiple-mutant genotypes were "germinated" in vitro, and the resulting plants were self-pollinated and ears sampled 20 DPP. Final confirmations of the multiple-mutant genotypes were based on the absence of the endosperm-specific BT1 polypeptide and activities of AGPase and Suc synthase in extracts of the freeze-dried 20-DPP endosperms. Dot blots of extracts from normal, *sh1*, and *sh2* were positive for polypeptides reacting with antibodies raised against BT1, but BT1 antibody-reacting proteins were completely absent from all blots of *bt1*-containing genotypes (data not shown). As expected, Suc synthase and AGPase activities were very low in all *sh1*- and *sh2*-containing genotypes, respectively (Table II). Soluble protein and activities of UGPase, starch synthase (soluble plus granule bound), and total SBE in the mutant genotypes were similar to those in normal kernels.

Kernel Characteristics of Normal and Single- and Multiple-Mutant Genotypes

Mature kernel phenotypes of the multiple-mutant genotypes are indistinguishable from *bt1* or *sh2* single-mutant kernels. Kernels of the double- and triple-mutant genotypes are viable. In this study we did not measure the endosperm dry weights of the various genotypes. However, we did determine the dry weights per endosperm of these same genotypes grown in the greenhouse in the late winter and spring of 1995 and harvested 20 DPP. The average dry weights per endosperm for normal and *sh1* were similar at 42.3 ± 5.9 and 41.8 ± 2.5 mg, respectively. Weights per endosperm of *sh2*, *bt1*, *sh1bt1*, and *sh2bt1* were similar at 34.6 ± 2.1 , 35.0 ± 2.7 , 34.7 ± 7.5 , and 32.5 ± 3.9 mg, respectively. Although these weights per endosperm are from plants grown in a different year from those ana-

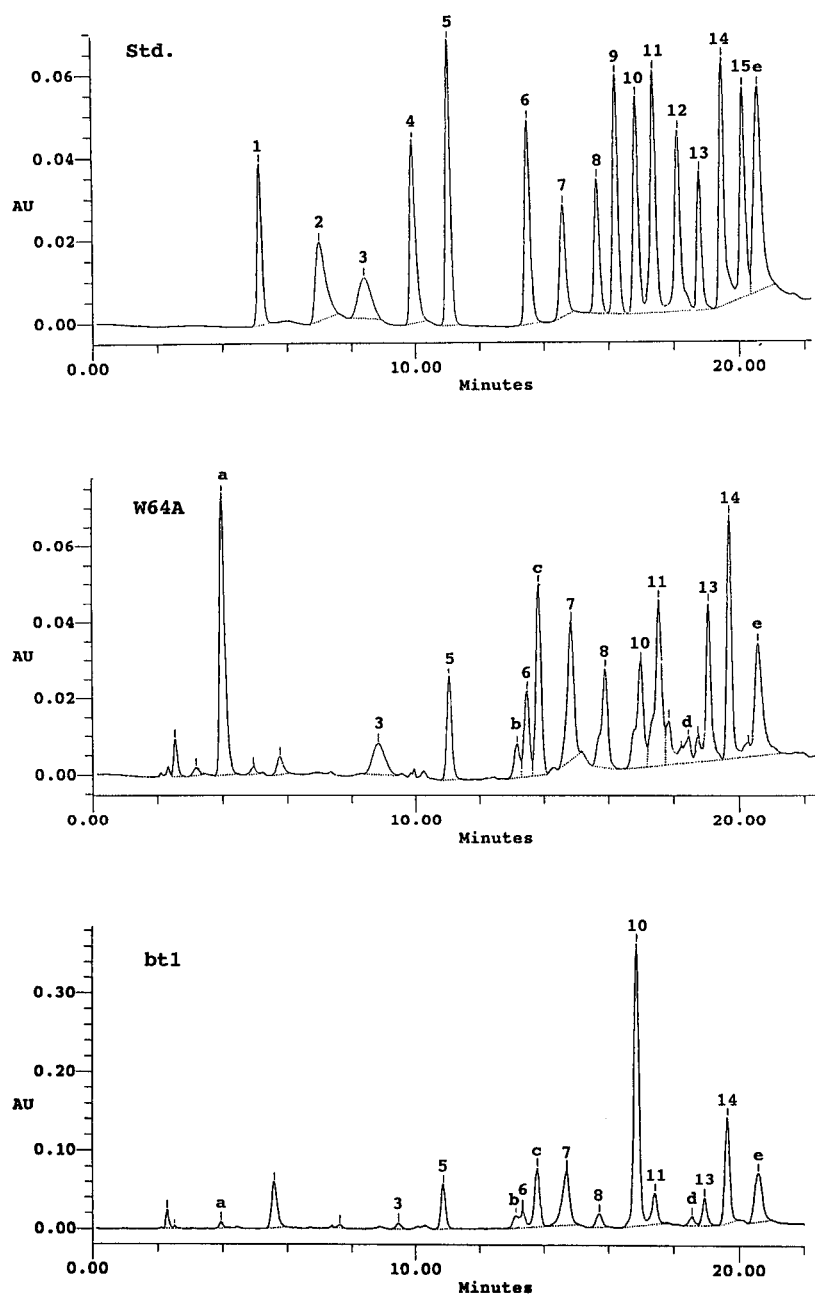


Figure 1. HPLC separation of nucleotide standards (top, Std.), endosperm extracts from the normal W64A inbred (middle), and endosperm extracts from the *bt1* mutant (bottom). A_{260} is plotted. Peaks are numbered according to the order of separation of the following standards: 1, uridine; 2, adenine; 3, UMP; 4, adenosine; 5, NAD; 6, AMP; 7, UDP-Glc; 8, UDP; 9, NADP; 10, ADP-Glc; 11, ADP; 12, NADH; 13, UTP; 14, ATP; 15, NADPH. Peaks a to e are unidentified. AU, Absorbance units of A_{260} .

lyzed in this study, we are safe in assuming that the relative weights per endosperm of normal (W64A) and the mutant genotypes will be similar, and thus these weights may be used to estimate content per endosperm. Suc and total reducing sugar contents in endosperms of the multiple-mutant genotypes were very high (Table III) as previously reported for the *sh2* and *bt1* genotypes (Tobias et al., 1992). Normal endosperms contained 2.14×10^6 starch granules mg^{-1} freeze-dried tissue ($90.5 \times 10^6/\text{endosperm}$) with an average of $293 \mu\text{g}$ starch 10^{-6} granules (Table III). The *sh1* endosperm contained about twice as many smaller starch granules as normal ($183.1 \times 10^6/\text{endosperm}$), whereas the *sh2*, *bt1*, *sh1bt1*, *sh2bt1*, and *sh1sh2bt1* en-

dosperm samples contained more than 4 times as many very small granules (Table III). The number of starch granules per endosperm for *sh2*, *bt1*, *sh1bt1*, and *sh2bt1* were estimated to be 604.5×10^6 , 466.2×10^6 , 370.2×10^6 , and 312.7×10^6 , respectively.

Nucleotide Quantity in Normal (W64A) Endosperm Cells

In normal endosperms the sum of uridine nucleotides was 1.7-fold higher than that of adenosine nucleotides. UTP was the highest of all nucleotides, and it was 2.4-, 1.8-, and 1.2-fold higher than UMP, UDP, and UDP-Glc, respectively (Fig. 2). Likewise, ATP was about 2.4-, 1.7-, and

Table I. Metabolite levels in normal (W64A) endosperm (20 DPP) and the percentage recovery of added standards

One-half gram samples of dry, pulverized endosperm from W64A kernels were extracted, and nucleotides were measured as described in "Materials and Methods." A mixture of nucleotides containing the indicated quantities were added to a second set of 0.5-g endosperm samples prior to extraction. Data are the averages \pm SD of two to eight extracts.

Metabolite	Endosperm Extract	Standard Added	Standard Recovered
	nmole/g dry wt	nmol/g dry wt	%
AMP	452 \pm 49	400	106 \pm 10
ADP	651 \pm 93	600	112 \pm 5
ATP	1097 \pm 62	1200	91 \pm 5
ADP-Glc	596 \pm 29	600	97 \pm 3
UMP	672 \pm 57	800	93 \pm 5
UDP	930 \pm 62	1000	105 \pm 2
UTP	1636 \pm 92	1600	97 \pm 5
UDP-Glc	1416 \pm 233	1400	101 \pm 11
NAD	391 \pm 22	400	98 \pm 7

1.8-fold higher than AMP, ADP, and ADP-Glc, respectively. The quantity of NAD⁺ was less than AMP (Fig. 2). The quantities of NADP⁺, NADH, NADPH, cytosine nucleotides, and guanidine nucleotides were either very low or below the level of detection.

Nucleotide Quantity in Endosperm Cells of Single Mutants

All mutant data are presented as percentages of nucleotide quantities in normal endosperm. The most striking effect of any of the three mutant genotypes was a 13-fold accumulation of ADP-Glc in the *bt1* mutant endosperm (Fig. 3). ADP-Glc per endosperm in the normal genotype was estimated to be 25.7 ± 1.3 nmol/endosperm, whereas contents per endosperm in *bt1*, *sh2*, and *sh1* were 271.1 ± 31.6 , 12.7 ± 2.4 , and 34.7 ± 4.0 nmol, respectively. The *bt1* endosperm extract was 2-fold higher in ATP and NAD and 3-fold higher in UDP-Glc but was lower in UMP, UDP, and UTP than extracts from normal endosperms (Fig. 3). Extracts from *sh2* endosperms were 4-fold higher in UDP-Glc but substantially lower than normal in UMP, UTP, and ADP-Glc. ADP, ATP, and NAD in *sh2* were higher than in normal endosperm. The content of nucleotides in *sh1* endosperm extracts was similar to that in normal endosperm with only ADP and ATP being substantially higher (Fig. 3).

The Effects of *Bt1* Dosage and Developmental Ages on the Quantity of Nucleotides

With each increase in the number of recessive *bt1* alleles, there was an increase in quantity of ADP-Glc and UDP-Glc, but the greatest increase occurred in homozygous recessive kernels (Table IV). All of the nucleotides in endosperms of W64A and *bt1* 10 DPP were much higher than those 20 DPP except for the ADP-Glc in *bt1*. Extracts from 10-DPP *bt1* endosperms contained 5.5 times more ADP-Glc than their normal counterparts, and by 20 DPP the ADP-Glc content had increased to a level more than 17 times higher than that in normal endosperms (Table IV).

Nucleotide Quantity in Endosperm Cells of Multiple-Mutant Genotypes

To determine whether Suc synthase or AGPase is responsible for the in vivo synthesis of ADP-Glc in *bt1*, we measured the nucleotides in extracts of *sh1bt1*, *sh2bt1*, and *sh1sh2bt1* endosperms. ADP-Glc in extracts of the *sh1bt1* double-mutant endosperms (deficient in Suc synthase) was similar to that in *bt1* (11.5-fold higher than normal), but in extracts of the *sh2bt1* double-mutant endosperms (deficient in AGPase) it was only about 3 times higher than that in normal endosperms (Fig. 4). Extracts of the *sh1sh2bt1* triple-mutant endosperms contained less than half as much ADP-Glc as extracts from normal endosperms. ADP-Glc contents per endosperm for *sh1bt1* and *sh2bt1* were estimated to be 239.6 ± 13.0 and 63.0 ± 0.7 nmol, respectively. If the kernel weight of the triple-mutant genotype is assumed to be the same as the average weight of the double-mutant genotypes, then the ADP-Glc content of *sh1sh2bt1* endosperms would be 9.4 ± 0.1 nmol/endosperm. By comparison, ADP-Glc contents per endosperm in normal and *bt1* endosperms were 25.7 and 271.1 nmol, respectively. UDP-Glc in extracts from all multiple-mutant genotypes was approximately 4-fold higher than in extracts from normal endosperms. The other uridine nucleotides were similar to or lower than in normal endosperms and, except for ADP-Glc in *sh1sh2bt1*, the adenosine nucleotides and NAD were equal to or higher than those in normal endosperm extracts (Fig. 4).

DISCUSSION

The mature kernel phenotypes of *bt1* and *sh2* are essentially identical, and the starch, neutral sugars, and hexose-P composition of extracts from developing *bt1* and *sh2* are

Table II. Protein and enzyme activities in normal and mutant maize endosperm

Genotype	Protein	AGPase	UGPase	Starch Synthase ^a	SBE	Suc Synthase
	$\mu\text{g mg}^{-1}$ dry wt			$\text{nmol min}^{-1} \text{mg}^{-1}$ dry wt		
Normal	46.6	10.3 \pm 0.6	55.0 \pm 16.4	0.7 \pm 0.0	132.1 \pm 23.2	22.2 \pm 7.9
<i>sh1</i>	48.5	12.3 \pm 1.7	52.9 \pm 20.2	1.1 \pm 0.0	177.8 \pm 37.6	1.6 \pm 0.3
<i>sh2</i>	53.6	1.8 \pm 0.1	53.9 \pm 10.4	1.7 \pm 0.0	221.9 \pm 37.9	21.9 \pm 4.0
<i>bt1</i>	49.8	12.1 \pm 0.3	35.2 \pm 0.2	1.6 \pm 0.1	179.2 \pm 45.1	16.3 \pm 5.8
<i>sh1bt1</i>	53.6	15.2 \pm 2.9	42.4 \pm 0.3	1.6 \pm 0.2	194.4 \pm 25.7	1.3 \pm 0.2
<i>sh2bt1</i>	59.9	1.8 \pm 0.5	55.1 \pm 3.6	1.6 \pm 0.1	222.3 \pm 33.5	19.5 \pm 0.4
<i>sh1sh2bt1</i>	57.9	1.4 \pm 0.2	44.6 \pm 4.7	1.8 \pm 0.2	207.5 \pm 39.9	0.7 \pm 0.1

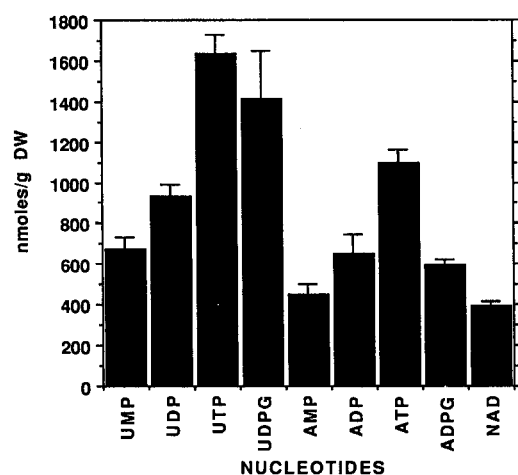
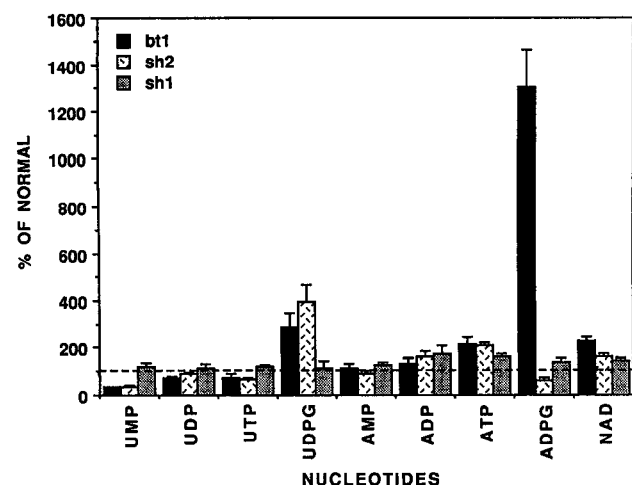
^a Starch Synthase, Sum of soluble and starch granule-bound activities.

Table III. Neutral sugars and starch granule number and quantity in extracts from 20-DPP normal (W64A) and mutant genotypes

Sugar data for the double mutants are the means \pm SE of duplicate extractions from the two composite samples, and data for the other genotypes are means \pm SE of duplicate extractions from composite samples.

Genotype	Suc	Reducing Sugars	Starch Granule Number	Starch Content
	nmol/mg dry wt		10^6 /mg dry wt	$\mu\text{g}/10^6$ granules
W64A	129.7 \pm 8.2	20.0 \pm 0.6	2.14	293
<i>sh1</i>	674.1 \pm 9.8	49.2 \pm 1.8	4.38	110
<i>sh2</i>	825.1 \pm 16.0	37.0 \pm 0.9	17.47	19
<i>bt1</i>	1114.4 \pm 23.0	48.0 \pm 0.9	13.32	19
<i>sh1bt1</i>	1077.0 \pm 58.6	48.8 \pm 3.7	10.67	21
<i>sh2bt1</i>	981.8 \pm 44.1	39.5 \pm 2.4	9.62	25
<i>sh1sh2bt1</i>	1198.8 \pm 42.2	67.2 \pm 0.9	9.36	20

very similar (Tobias et al., 1992). In contrast, ADP-Glc content in *sh2* is reduced relative to normal, whereas that in *bt1* is 13- to 17-fold higher than in normal endosperm extracts (Fig. 3; Table IV). Quantities of the other nucleotides in *sh2* and *bt1* were similar. Two enzymes, AGPase and Suc synthase, are capable of synthesizing ADP-Glc in vitro. The *sh2* mutant is deficient in AGPase (Tsai and Nelson, 1966; Dickinson and Preiss, 1969), and *sh1* is deficient in Suc synthase (Chourey and Nelson, 1976). To determine which enzyme is responsible for the in vivo synthesis of ADP-Glc in *bt1* kernels, we produced the multiple-mutant genotypes *sh1bt1*, *sh2bt1*, and *sh1sh2bt1* and determined the content of the various nucleotides. ADP-Glc in endosperm extracts of the *sh1bt1* double mutant was similar (11.5-fold higher than normal) to that in *bt1*, but the endosperm extract from *sh2bt1* was only about 3 times higher in ADP-Glc than normal endosperm extracts (Fig. 4). Extracts of the *sh1sh2bt1* triple-mutant endosperms contained less than half as much ADP-Glc as extracts from normal endosperms. We conclude from these results that AGPase is the predominant enzyme responsible for the in

**Figure 2.** Quantity of nucleotides in extracts from normal endosperms (W64A). Data are the means \pm SE of four extractions. UDPG, UDP-Glc; ADPG, ADP-Glc; DW, dry weight.**Figure 3.** Quantity of nucleotides in endosperm extracts from the single-mutant genotypes *bt1*, *sh2*, and *sh1* presented as percentages of the quantity in normal endosperms. UDPG, UDP-Glc; ADPG, ADP-Glc.

vivo synthesis of ADP-Glc in *bt1* mutant kernels. However, it appears that Suc synthase may also play a role in the in vivo synthesis of ADP-Glc, because the extract of *sh1sh2bt1* endosperm, which is deficient in both AGPase and Suc synthase, was much lower in ADP-Glc than extracts of *sh2bt1* or normal endosperms.

AGPase has been reported to be localized in the amyloplast stroma of potato tubers (Kim et al., 1989) and maize endosperms (Miller and Chourey, 1995) and in the stroma of chloroplasts (Preiss, 1991). Localization of AGPase in plastids is generally accepted (Okita, 1992; Smith et al., 1995). Extracts of *bt1* endosperms contain activities of the enzymes for synthesis (AGPase) and utilization (starch synthase) of ADP-Glc that are equal to or higher than those in extracts from normal endosperms (Table II). However, starch accumulation in *bt1* kernels is only 20% of that in normal kernels (Tobias et al., 1992). If ADP-Glc is synthesized in the amyloplast stroma by a plastid-localized AGPase and the activity of starch synthase, another plastid-localized enzyme, is not limiting, then why does the ADP-Glc accumulate rather than being utilized in the synthesis of starch? Synthesis of starch by starch synthase requires the presence of an oligosaccharide primer in addition to ADP-Glc (Preiss, 1991). UPTG (EC 2.4.1.112) is a specialized protein in potato tubers (Lavintman et al., 1974) and maize endosperm (Cura et al., 1994; Rothschild and Tandecarz, 1994; Tandecarz et al., 1995), which functions in the initiation and elongation of the oligosaccharide primer required for de novo synthesis of starch (Cura et al., 1994; Tandecarz et al., 1995). Maize UPTG is a multimeric protein composed of identical 38-kD subunits. The nuclear-encoded BT1 polypeptides (Sullivan et al., 1991) are of a similar size (39–44 kD) (Cao et al., 1995). BT1 polypeptides have been shown by immunocytochemical localization (Sullivan and Kaneko, 1995) and by studies of isolated amyloplast membranes (Cao et al., 1995) to be specifically localized to the amyloplast membranes of developing maize endosperm. BT1 accounts for about 40% of the

Table IV. The effects of *Bt1* gene dosage and kernel age on the quantity of nucleotides in maize endosperm

Total endosperm (0.5 g dry weight) was extracted and nucleotides measured as described in "Materials and Methods." Values are means \pm SE of two replications.

Endosperm Genotype	Kernel Age	ADP	ATP	ADP-Glc	UMP	UDP	UTP	UDP-Glc	NAD
	DPP	nmol/g dry wt							
<i>Bt1Bt1Bt1</i>	20	456 \pm 26	1525 \pm 103	482 \pm 8	428 \pm 27	924 \pm 70	1425 \pm 139	1232 \pm 80	547 \pm 33
<i>Bt1Bt1bt1</i>	20	470 \pm 10	1738 \pm 6	518 \pm 2	701 \pm 12	984 \pm 1	1400 \pm 27	1380 \pm 6	590 \pm 2
<i>Bt1bt1bt1</i>	20	408 \pm 1	1695 \pm 24	581 \pm 12	568 \pm 27	906 \pm 16	1435 \pm 41	1432 \pm 24	543 \pm 10
<i>bt1bt1bt1</i>	20	498 \pm 20	2273 \pm 120	8447 \pm 294	348 \pm 4	654 \pm 24	315 \pm 20	3108 \pm 120	1212 \pm 39
<i>Bt1Bt1Bt1</i>	10	1204 \pm 50	4323 \pm 96	709 \pm 6	1058 \pm 11	1642 \pm 26	4588 \pm 80	3514 \pm 124	690 \pm 6
<i>bt1bt1bt1</i>	10	1252 \pm 98	4800 \pm 206	3932 \pm 185	1026 \pm 49	1692 \pm 62	4027 \pm 177	4384 \pm 180	1022 \pm 43

polypeptides associated with amyloplast membranes from normal endosperms but is missing from amyloplast membranes isolated from *bt1* mutant kernels (Cao et al., 1995). A possible explanation for the ADP-Glc accumulation in *bt1* kernels is that *Bt1* encodes maize UPTG and in its absence de novo starch synthesis is reduced because of a deficiency in oligosaccharide primer. Although this is an attractive possibility, it was not supported by recent evidence from Tandecarz's laboratory. They showed that extracts from endosperms of both W64A (wild type) and *bt1* contained proteins that reacted with antibodies raised against the 38-kD potato tuber UPTG protein (A. Rothschild, S. Bocca, and J. Tandecarz, unpublished results). In addition, our observation of the large number of small starch granules in *bt1* endosperms does not support the suggestion that *Bt1* encodes synthesis of UPTG.

A second possible explanation for the high ADP-Glc in *bt1* endosperm cells is that the ADP-Glc accumulates in a compartment physically separated from starch synthase. Since starch synthase is localized in the plastid stroma (Echeverria et al., 1988), it follows that in *bt1* endosperm cells ADP-Glc accumulates in the cytosol. Kleczkowski et al. (1991) and Hannah et al. (1993) suggested that cereal endosperms may contain AGPase isozymes in the cytosol

as well as in the amyloplast stroma, and Villand and Kleczkowski (1994) detailed the evidence in support of this suggestion. A possible explanation is that BT1 is an adenylate translocator, and in its absence (i.e. in *bt1* kernels) ADP-Glc synthesized in the cytosol is unable to enter the amyloplasts and thus it accumulates. This suggestion is supported by the observation that amyloplasts isolated from immature *bt1* endosperm were only 25% as effective in the uptake and incorporation of ADP-Glc into starch as amyloplasts isolated from normal or *wx* endosperms (Liu et al., 1992). In addition, Sullivan et al. (1991) reported that the *Bt1*-encoded protein is most closely related to a yeast adenylate translocator.

A third possible explanation for the ADP-Glc accumulation in the cytosol of *bt1* kernels is that BT1 may be the protein transporter required for the transfer and processing of the AGPase subunits into the amyloplast stroma. According to this suggestion the AGPase subunit transfer would not occur in *bt1* mutant endosperm cells, and instead the AGPase subunit(s) would remain in the cytosol where it is processed into active AGPase. Ballicora et al. (1995) reported that the small subunit of potato AGPase expressed in *Escherichia coli*, without the large subunit, exhibited high enzyme activity, but the regulatory properties differed from those of the heterotetrameric enzyme expressed in *E. coli*. If we assume that the maize amyloplast membrane is unable to transfer ADP-Glc, as in potato tubers (Stark et al., 1992), then accumulation of ADP-Glc in the cytosol of *bt1* endosperm cells would occur. Further studies are needed to conclusively determine the function of BT1.

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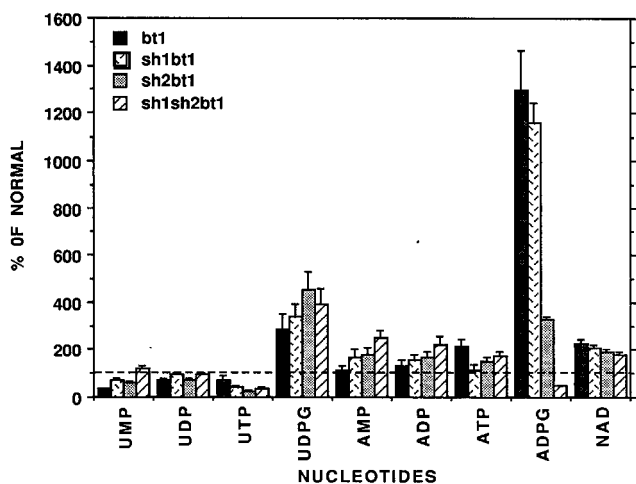


Figure 4. Quantity of nucleotides in endosperm extracts from *bt1* and the multiple-mutant genotypes *sh1bt1*, *sh2bt1*, and *sh1sh2bt1* presented as percentages of the quantity in normal endosperms. UDPG, UDP-Glc; ADPG, ADP-Glc.

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